Research

Working memory deficits in retinoid X receptor γ-deficient mice

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Retinoid signaling has been recently shown to be required for mnemonic functions in rodents. To dissect the behavioral and molecular mechanisms involved in this requirement, we have analyzed the spatial and recognition working memory in mice carrying null mutations of retinoid receptors RARβ and RXRγ. Double mutants appeared deficient in spatial working memory as tested in spontaneous alternation in the Y-maze and delayed nonmatch to place (DNMTP) test in the T-maze. These mutant mice did acquire, however, spatial place reference or right/left discrimination tasks in the T-maze set-up, indicating that basic sensorimotor functions, spatial orientation, and motivational factors are unlikely to account for deficits in working memory-sensitive tasks. Double-mutant mice were also deficient in novel object recognition at intermediate, but not short delays. RXRγ appeared to be the functionally predominant receptor in modulation of the working memory, as RXRγ, but not RARβ single null mutant mice exhibited deficits similar to those observed in the double mutants. The mechanism of this modulation is potentially related to functions of RXRγ in frontal and perirhinal cortex, structures in which we detected RXRγ expression and which are functionally implicated in working memory processes.

Retinoic acid (RA, the major active form of vitamin A) is involved in the control of functions of several adult organs, including brain. Diet-induced or age-related reduction of retinoic acid levels was recently reported to lead to mnemonic deficits in spatial learning and memory in rats (Cocco et al. 2002) or relational memory in mice (Etchamendy et al. 2001, 2003). In view of the pleiotropic effects of RA, several dysfunctions may be involved in the generation of these deficits. Thus, one of the first steps in elucidating the molecular mechanisms underlying the modulation of mnemonic functions by retinoids is to determine which RA-signaling pathway(s) is implicated in this control.

In vertebrates, the retinoid signal is mediated by two families of nuclear receptors, the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs), each family comprising three isotypes, α β, and γ with several isoforms for each isotype (Chambon 1996). These receptors are ligand-dependent transcriptional regulators acting as RAR/RXR heterodimers (Chambon 1996; Kastner et al. 1997). As RARs and RXRs are expressed in the adult mouse brain (Krezel et al. 1999; Zetterstrom et al. 1999) they can be directly implicated in control of brain functions. The concomitant null mutation of RARβ and RXRγ or the null mutation of RARβ alone have been linked with spatial long-term memory deficits observed in the place-reference version of the Morris water-maze task (Chiang et al. 1998). Whether the inability of RARβ/RXRγ double mutants to learn this task could be related to deficits in the working memory, which actually would be the primary origin of deficient performance of these mutants in the water-maze task, was not investigated in this latter study. Also, some of the locomotor deficits, which result from inactivation of RARβ and are present in RARβ/RXRγ double-mutant mice (Krezel et al. 1998) could affect swimming capabilities, thus contributing to deficient performance in the water maze.

To investigate the specificity and mechanisms of RA modulation of mnemonic functions, we have now studied the effect of the RARβ and RXRγ loss-of-function on working memory in mice. To avoid the possible functional redundancies that could occur among retinoid receptors when a single receptor is knocked out (Kastner et al. 1997; Krezel et al. 1998), we initially studied in detail RARβ/RXRγ double-null mutant mice, which then allowed us to select the tests to be performed on single null mutant mice to identify the role played by individual receptors.

Results

Behavioral studies of RARβ and RXRγ double and single null mutants

Spatial working memory in the delayed nonmatch to place in T–maze

To investigate the spatial working memory, we have first chosen delayed nonmatch to place task, as equivalent tasks are used in clinical conditions and were extensively validated with lesion studies in rodents and nonhuman primates. One double mutant was excluded from the analysis, as it did not move in the maze on the two final days of training. The minimal, "0 sec", interval between the acquisition, "forced run", and the retention, "choice run", was sufficient to reveal the main effect of genotype [$F_{(1,30)} = 44$, $P < 0.001$] and significant differences in the evolution of the learning curves between wild-type and RARβ/RXRγ mutant mice as reflected by significant interaction between the genotype and the day [$F_{(8,240)} = 8.8$, $P < 0.001$]. Post-hoc analysis showed that wild-type mice performed significantly better ($P < 0.001$) than their mutant counterparts during the last 3 d of training and attained 90% of correct choices as compared with 64% in the mutant group (Fig. 1A). Nevertheless, the mutant

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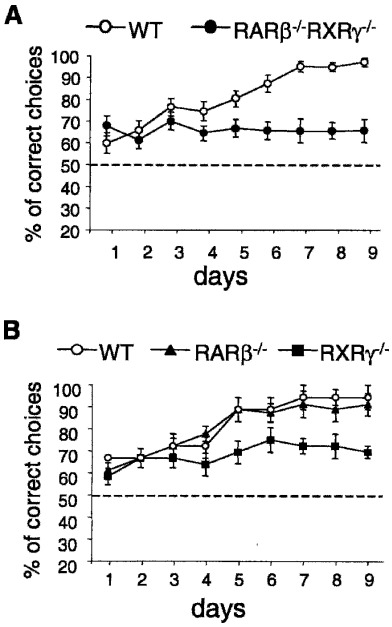

Figure 1. Performance of RARβ and/or RXRγ mutants in the delayed nonmatch to place task. For each testing day, choice accuracy was represented as the percent of correct choices ± SEM for (*A*) WT and RARβ/RXRγ double-mutant mice and (*B*) single RARβ and RXRγ mutants with respective control group.

mice performed above the chance level of 50% ($P < 0.05$, one group *t*-test). Further analysis revealed significant increase of latencies of double-mutant mice in the DNMTP, reaching, on the last day, 8.9 ± 0.9 sec for double-mutant mice and 2.9 ± 0.2 sec for the wild-type group with the main group effect for genotype [$F_{(1,30)} = 17$, $P < 0.001$].

The poor performance of RARβ/RXRγ double mutants could not result from the spontaneous perseverant behavior, as intra-animal analysis of errors did not reveal any laterality in animal choices, as only three of 15 mutant and two of 17 control mice persistently (two times per day or more) committed errors by turning to the left or right arm during any three consecutive days of testing. Similarly, we have not found any significant "procedural" perseverance, such as re-entering arms visited in the acquisition phase (forced run), as none of the mutant or control animals showed 83% or more errors on any three consecutive days of testing. Finally, animal behavior was not biased with respect to any external or internal cues in the present experimental setting, as the percent of errors committed by entrance to the left arm was 51 ± 5% of total errors for wild-type and 49 ± 6% for mutant mice.

Single-mutant mice were analyzed to evaluate the contribution of each receptor to the deficits observed in RARβ/RXRγ double-mutant mice. The main effect of the genotype [$F_{(2,18)} = 13.8$, $P < 0.001$] for reduced number of correct choices was observed in RXRγ null mutant mice, which reached 70%, and which was significantly lower than 93% for wild-type or 89% for RARβ mutants over the last 3 d (at least $P < 0.01$ for each individual day; Fig. 1B). Similarly to double-mutant mice, the performance of single-mutant RXRγ mice was significantly different from chance level of 50% of correct choices ($P < 0.05$, one group *t*-test). However, unlike for double-mutant mice, the latencies of RARβ or RXRγ single mutants were not different from their wild-type controls, and during the choice runs on the last day, attained, on average, 5.5 ± 1.1 sec, 3.2 ± 0.3 sec, and 3.4 ± 0.4 sec, respectively.

Place reference and right/left discrimination in the T-maze

As an inability to navigate in the experimental space could be at the origin of deficient performance in delayed nonmatch to place in the T-maze, we investigated whether mutant mice can develop strategies to orient themselves in space according to extra-maze cues (allocentric strategy) or their own body position (egocentric strategy).

All double-mutant mice analyzed in forced choice alternation were tested for spatial learning and memory in the place-reference test in the same room and apparatus. One-week intervals were introduced between each of the consecutive tasks, and animals were not tested during this time. We found that both double-mutant mice and their controls had learned this task, and after 9 d of training, made 82% or 90% of correct choices, respectively (Fig. 2A). We noticed, however, that although not statistically significant, [$F_{(8,240)} = 1.92$, $P = 0.058$] mutant mice showed a tendency to learn this task more slowly. Cumulative probability analysis showed that this tendency was representative for the entire group, rather than due to low performance of a few individual mice, and thus, on the last day of the test, the probability that any given mutant mouse performs below 90% of correct choices was 0.65 as compared with 0.2 for wild-type controls (Fig. 2A, right).

All mice previously trained in the forced-choice alternation and place-reference task were also tested in the right/left discrimination task, which was carried out in the same room and experimental set up. Both double-mutant and wild-type mice acquired this task equally well and performed at 86% and 83% of correct choices on the ninth day of the test (Fig. 2B). However, in contrast to the place preference task, the probabilistic performance of each group was comparable (Fig. 2B, right).

Finally, to eliminate proactive interference among different tests carried out on the same groups of animals, we prepared two naive groups of wild-type ($n = 7$ for each procedure) and RARβ/RXRγ ($n = 7$ for each procedure) mutant mice, and tested them independently on the place reference or right/left discrimination test. The performance of RARβ/RXRγ mutants and wild-type mice showed comparable learning curves to those illustrated in Figure 2, A and B, respectively (data not shown).

As no statistical differences were observed in these tasks, we did not carry out additional tests on single-mutant mice.

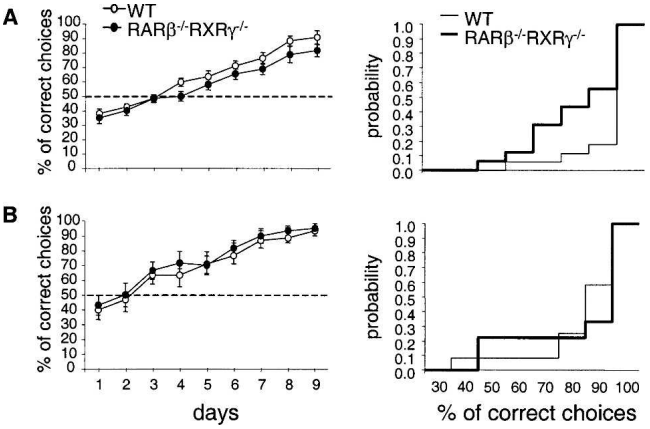

Figure 2. Performance of RARβ/RXRγ double null mutant mice in the cross-maze tasks for allocentric and egocentric spatial learning and memory. The percent of choice accuracy was expressed as means ± SEM for WT and RARβ/RXRγ double mutants for each testing day in the place reference (*A*, *left*) and right/left discrimination (*B*, *left*) tests. The cumulative probabilities of the percent of correct choices were calculated for the ninth day of the experiment (*right* side of the corresponding performance curves).

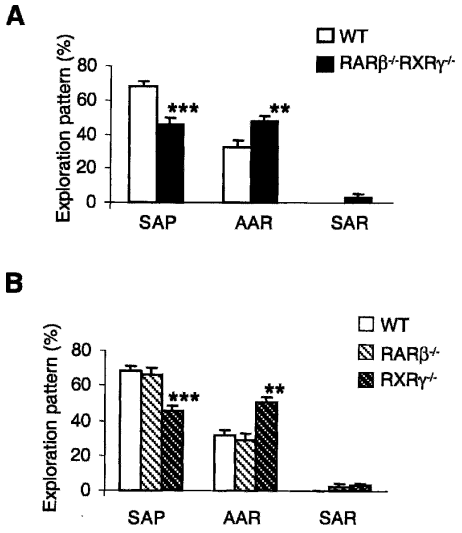

Figure 3. Effects of RARβ and/or RXRγ mutation(s) on the spontaneous exploration in the Y-maze. The percent of spontaneous alternation performance (SAP), alternate arm returns (AAR), and same arm returns (SAR) ± SEM were represented for RARβ/RXRγ double mutants (*A*), and for corresponding single mutants (*B*). Statistical differences with respect to WT control animals were indicated. (**) $P < 0.01$; (***) $P < 0.001$.

Spatial working memory in the spontaneous alternation task in Y–maze

The procedural memory requirements, the stress (behavioral and metabolic) related to experimental procedures, including water deprivation, could nonspecifically affect animal performance in delayed response tasks. Thus, to further assess the specificity of spatial working memory deficits related to the loss of RXRγ function, we have chosen to test spontaneous alternation in the Y-maze, which is devoid of all these procedural aspects, as it is based on the natural tendency of mice to explore novel environment. All animals showed good ambulatory activity (above six entries), and the exploration rates were not affected by double mutation, as the total number of arm entries ranged at 19.7 ± 1.6 and 16.8 ± 1.2 for wild-type and RARβ/RXRγ mutant groups. Double-mutant mice displayed spontaneous alternation of 45.5% ± 4% of choices, which was significantly lower than 60.7% ± 2.9% of SAP for the wild-type control group ($P < 0.05$, student *t*-test; Fig. 3A). The analysis of single mutants revealed a significant effect of the genotype [$F_{(2,33)} = 15.5$, $P < 0.001$], which was due to lower, 45.9% ± 2.8% alternation rates of RXRγ mutant mice as compared with 68% ± 3% for wild-type and 66.5% ± 3.6% for RARβ group ($P < 0.001$ for RXRγ vs. wild-type or RARβ; Fig. 3B). We have also observed a correlation between reduced alternation rates and increase in alternate arm returns, but not the same arm returns. Thus, there was a significant increase ($P < 0.01$) of the AARs exclusively in double-mutant (47.1% ± 3.7%; Fig. 3A) and RXRγ single-mutant mice (50.2% ± 3.1%; Fig. 3B) as compared with their respective wild-type controls (32.5% ± 3.5% and 31.5% ± 2.9%).

Object–recognition test

Behaviorally naive wild-type and RARβ/RXRγ mutant mice were first habituated to the experimenter and the experimental set up to reduce the contaminating effects of affective influences in mnemonic assessment. When tested in the object-recognition test, the retention intervals were set at 1, 3, and 24 h to obtain a wider range of sensitivities of this test to working memory and to see performance at chance level with 24-h delays (Meziane et al. 1998). The exploratory behavior of wild-type and double-mutant mice was not different during the acquisition phase, and controls explored object A for 11.9 ± 0.8 sec and mutants for 10.47 ± 0.9 sec. When the interval between the acquisition phase and the retention test was low (1 h), both wild-type and mutant mice showed good short-term memory. This was illustrated by more time spent exploring the new object than the familiar one during the retention trial, which attained 69% ± 3% of total exploration time for wild-type and 68% ± 3% for RARβ/RXRγ mutant mice (Fig 4A). In contrast, extension of the interval to 3 h revealed that mutant mice could not distinguish between familiar and novel objects as effectively as control mice, and explored the new object for 58% ± 4% of the total exploration time as compared with 69% ± 4% for wild-type controls ($P < 0.05$, Fig 4A). This test is characterized by rapid forgetting. Thus, after 24 h, as expected, wild-type and mutant animals did not distinguish between familiar and novel object and explored each of them for equal lengths of time (Fig. 4A).

To investigate the individual contribution of each receptor in the generation of recognition memory deficit displayed by RARβ/RXRγ mutants, we analyzed RARβ and RXRγ single-mutant mice in the object recognition test. We found that the significant effect of the group for genotype [$F_{(2,36)} = 13.7$, $P < 0.001$] is related to low performance of only RXRγ mutants, which could not discriminate between familiar and novel objects after 3 h of retention and explored the new object for 56% ± 3% of the total exploration time as compared with 69.9% ± 5% for wild-type and 72% ± 3% for RARβ mutant mice ($P < 0.001$ RXRγ vs. wild-type or RARβ; Fig. 4B). This reduced capacity for retention of the information in RXRγ mutant animals was comparable to that observed in double mutants (Fig. 4, cf. A and B). These data support the predominant role played by RXRγ, not only in spatial, but also recognition working memory in mice.

Study of cortical localization of RXRγ

Thorough examination of immunohistological preparations confirmed previously reported expression of RXRγ in the hippocampus (Zetterstrom et al. 1999), showing, in addition, that RXRγ was restricted to only a few cells in the polymorphous layer of the dentate gyrus in the dorsal part, whereas in the ventral part of hippocampus, weak labeling was also present in the CA3 region.

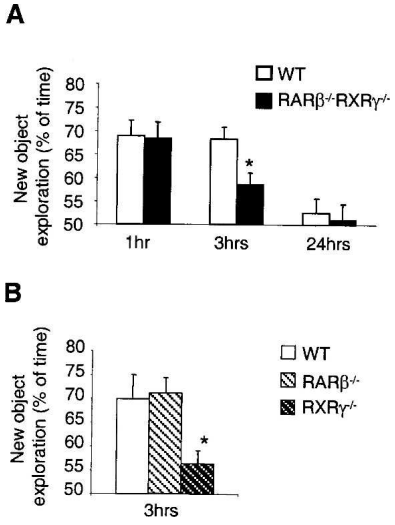

Figure 4. Object recognition in RARβ and/or RXRγ null mutant mice. The time of exploration of the novel object was expressed as percent of the total exploration time of both objects and was used as a measure of the working memory in behaviorally naive groups of WT and RARβ/RXRγ double mutants (*A*), and RARβ and RXRγ single-mutant mice with respective WT controls (*B*). (*) Significantly different from controls ($P < 0.05$).

We have found, however, new, not previously reported regions of expression of RXRγ. In particular, a subpopulation of cells within infralimbic and prelimbic cortices displayed high-to-moderate levels of RXRγ (Fig. 5A), as compared with dorsal striatum, one of the sites of the strongest expression of this receptor. These parts of the medial prefrontal cortex receive reciprocal connections with other cortical regions, including perirhinal and entorhinal cortex, as well as mediodorsal thalamic nucleus and a number of other subcortical regions, including amygdala or afferent pathway from the hippocampus (Groenewegen et al. 1997; Uylings et al. 2003). The RXRγ was also detected in perirhinal cortex (Fig. 5B). This region being a part of the parahippocampal pathway is thought to be the major source of afferent sensory information to the hippocampus via entorhinal cortex or via direct projections to the hippocampus (Witter et al. 1989; Burwell et al. 1995).

Discussion

We report here that concomitant null mutations of RARβ and RXRγ or a null mutation of RXRγ alone lead to deficits in spatial and recognition working memory in mice. These deficits were apparent in distinct behavioral tasks, i.e., the delayed nonmatch to place and spontaneous alternation tests sensitive to spatial memory and novel object recognition test for recognition memory. The RARβ/RXRγ or RXRγ null mutant mice displayed severe deficit in the delayed nonmatch to place task, even with the shortest, "0"-sec delays. These mutant animals, however, were capable of solving egocentric, right/left discrimination or allocentric place reference tasks in the same experimental setting, thus proving that sensorimotor functions, spatial orientation, and motivational factors critical for performance in the T-maze are unlikely to account for the deficits in the DNMTP task. The spontaneous or procedural perseverance, which could similarly lead to such deficits and was previously associated with specific cortical dysfunctions (Ragozzino et al. 1999b), was ruled out by an intragroup analysis of errors (see Results) and by intact behavioral flexibility during a cross-modal switch between different spatial discrimination tasks in the T-maze (Fig. 2B). We have noticed, however, that performance of RARβ/RXRγ or RXRγ mutants in DNMTP attained almost 70% of correct choices and was significantly higher than chance level of 50%, which might illustrate residual working memory or suggest that reduced performance in DNMTP is related to procedural complexity of this test, and thus result from, e.g., behavioral and/or metabolic stress of water deprivation, reduced procedural memory or proactive interference between consecutive runs, and/or trials. To exclude these procedural components, we have tested mutant mice for spontaneous alternation in the Y-maze, the working memory test based on a natural tendency of mice to explore a novel environment. Only RARβ/RXRγ and RXRγ mutants showed reduced spontaneous alternation as compared with wild-type or RARβ mutant mice. However, performance of all mutant mice remained above the chance level, which for Y-maze performance, is calculated at 22.2%. Furthermore, the error analysis showed that reduced performance in the Y-maze correlated for double- and RXRγ single-mutants with a higher number of alternate arm returns and not with the same arm returns, thus being a further indication of reduced attention/working memory in these mutant animals (Holcomb et al. 1999; Wall and Messier 2002). Thus, the reduction of spontaneous alternation indicates that in agreement with results from DNMTP test, an inactivation of RXRγ alone or in parallel with RARβ leads to reduction, but not complete absence of spatial working memory.

It is also noteworthy that although we did not observe any consistent statistical differences between mutant and control mice in the place-reference task in the T-maze, the mutant mice showed a tendency for reduced ability to acquire this task, which is best seen on the cumulative probability curve for the place-reference test (Fig. 2A). This tendency could become statistically significant, possibly with less intense learning protocol. Yet, we have chosen a 12-trial protocol to retain the maximum of procedural similarities with the delayed nonmatch to place, in order to facilitate its interpretation. In contrast to our data showing only a slight retardation in solving the place reference paradigm, RARβ/RXRγ and also RARβ, but not RXRγ mutants were reported to have substantial deficits in spatial navigation in the Morris water-maze set-up (Chiang et al. 1998). This discrepancy could be due to the aversive character of the water-maze or the reduced locomotor skills of RARβ/RXRγ and RARβ mutants (Krezel et al. 1998), which are critical for swimming in the water maze and may confound the interpretation of the results of this test. Thus, to reduce the aversive character of behavioral testing, we used appetitive tasks. Furthermore, despite longer latencies of RARβ/RXRγ double mutants in the DNMTP test, locomotor problems are unlikely to lead to deficient performance in present spatial working memory tasks since: (1) RXRγ mutation led to deficits in the DNMTP performance without affecting latencies in this task, (2) RARβ/RXRγ and RXRγ mutations reduced spontaneous alternation in the Y-maze without altering the number of arm entries. Thus, as our RARβ/RXRγ mutant mice were capable of acquiring different T-maze tasks with exception to DNMTP and displayed

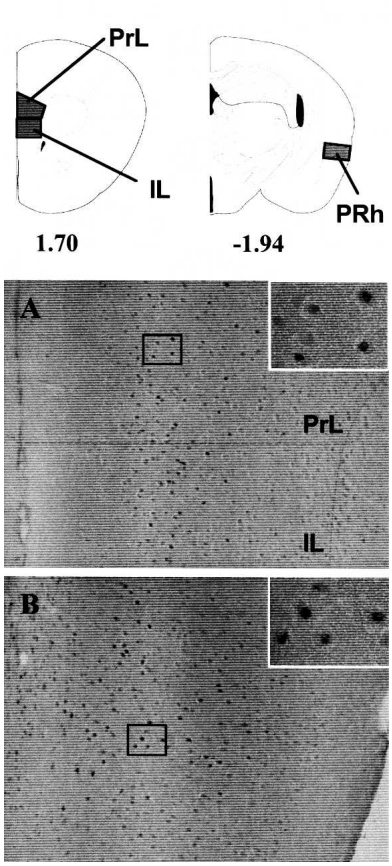

Figure 5. Immunolocalization of RXRγ protein in the mouse cortex. The diagrams of coronal sections of prelimbic (PrL), infralimbic (IL), and perirhinal (PRh) cortex with distance (mm) of the section from bregma (Paxinos and Franklin 2001) were shown at *top*. The immunodetection of RXRγ in IL and PrL (A) and PRh (B) cortex were presented at × 40 magnification. The nuclear staining in selected regions (boxes) was presented at × 400 magnification in the *top, right* corner of each panel.

decreased rates of spontaneous alternation in the Y-maze, we conclude that their reduced efficiency of solving the place-reference paradigm in the T-maze may be a consequence of, and therefore secondary to, compromised spatial working memory related to dysfunction of RXRγ. Finally, deficits in working memory may be at the origin of spatial or relational memory deficits observed in rodents during aging or with reduced levels of RA (Etchamendy et al. 2001, 2003; Cocco et al. 2002).

Working memory deficits observed in RARβ/RXRγ or RXRγ null mutant mice extend also into recognition memory, as tested in the object-recognition test, which has been proposed to be a rodent equivalent of the one-trial nonmatching to the sample test used in monkeys (Ennaceur and Delacour 1988). Consistent with that idea, the object-recognition task, which assesses a form of novelty-motivated working memory for visual objects, showed time-window dependence, as wild-type mice displayed good retention at 1- and 3-h, but not 24-h delays. The deficits in object discrimination that we observed at 3-h delays in RARβ/RXRγ or RXRγ mutants may result from reduced duration of the memory, as mutant mice were clearly capable of acquiring, analyzing, and retaining this information for a shorter period of 1 h.

The difference in the severity of the delay-dependent mnemonic deficits that we observe in our mutants in object recognition and delayed nonmatch to place is most probably directly related to differences between the two paradigms, including possible differences in anatomical substrates critical for solving these tasks. Thus, spatial delayed tasks have been shown to implicate frontal cortical (Goldman-Rakic 1999; Levy and Goldman-Rakic 2000; Fuster 2001) and to limited extend hippocampal functions (Mishkin 1978; Winocur 1992; Holdstock et al. 1995; Steele and Morris 1999; Eichenbaum 2000; Lee and Kesner 2003) in primates and rodents. In fact, there is a striking similarity between the effects of lesions of the frontal cortex or discrete concomitant lesions of frontal cortex and hippocampus (Dias and Aggleton 2000; Lee and Kesner 2003) and deficits displayed by our mutant mice in DNMTP performance. The synaptic plasticity of hippocampal afferents in the frontal cortex has been suggested relevant for functional relaying of these two regions during working memory (Laroche et al. 2000) and might be a target of RXRγ modulations, as RXRγ is expressed in both of these regions and, at least in the hippocampus, has been shown to modulate long-term depression (Chiang et al. 1998). The involvement of frontal cortex and hippocampus in object-recognition memory is less clear, as lesion studies provided both positive (Steckler et al. 1998; Clark et al. 2000; Ragozzino et al. 2002; Hammond et al. 2004) and negative evidence (Ennaceur et al. 1997; Baxter and Murray 2001; Mumby 2001; Gaskin et al. 2003). In contrast, excitotoxic lesions (Aggleton et al. 1997; Bussey et al. 2000; Liu and Bilkey 2001) or studies of neuronal activities (Brown and Aggleton 2001, and references therein) provided more solid evidence for implication of the parahippocampal region, including perirhinal cortex in visual object-recognition memory. Since perirhinal cortical lesions do not affect spatial memory (Bussey et al. 2000; Winters et al. 2004), we speculate that RXRγ expressed in this region (Fig. 5) may be implicated in the control of cortical functions relevant to recognition memory. Finally, we also hypothesise that the novelty-related reinforcement of memory proposed by Tulving and Kroll (Tulving 1995), can potentially account for the relatively better performance of RARβ/RXRγ mutants in the object recognition test than in the delayed nonmatch to place task. An exposure to novelty during learning has been correlated with increased activities in the hippocampus in humans (Tulving et al. 1996) and rats (Vann et al. 2000) and was suggested to involve dopamine-mediated facilitation of long-term potentiation in the hippocampus (Li et al. 2003).

The dopaminergic signaling has long been suggested to control working memory processes, and abnormal dopamine signaling in the prefrontal cortex was demonstrated to cause spatial working memory deficits in delayed-response tasks in nonhuman primates and rodents (Goldman-Rakic 1999), and in humans has been associated with schizophrenia (Laruelle et al. 2003; Goldman-Rakic et al. 2004). In fact, retinoids appear an important regulator of dopaminergic signaling. We have previously shown that in the striatum RARβ and RXRγ are the key retinoid receptors involved in modulation of expression of dopaminergic receptors (Krezel et al. 1998), which may take place at the transcriptional level, as dopamine D2 receptor contains a functional retinoic acid regulatory element in its promoter (Samad et al. 1997). Although dysfunction of dopaminergic signaling is a potential mechanism of spatial working memory deficits observed in our mutant mice, we cannot exclude the implication of other neurotransmission systems in this phenotype. Furthermore, the deficits in recognition memory are not easily explained by altered dopamine signaling, as dopaminergic modulation of working memory for recognition of visual objects is less clear (Besheer et al. 1999). Cholinergic neurotransmission is another potential mediator of retinoid modulations of working memory, as expression of choline acetyl transferase (ChAT) and vesicular acetylocholine transporter can be modulated by RA treatment (Berrard et al. 1995; Berse and Blusztajn 1995) and vitamin A deficiency or RXRγ1 inactivation were associated with reduced expression of ChAT (Saga et al. 1999; Cocco et al. 2002; Corcoran et al. 2004). Indeed, reduced cholinergic signaling is associated with Alzheimer disease, for which working memory deficits are some of the early symptoms. The circuits involving hippocampus, prefrontal, or perirhinal cortices are some of the key sites of cholinergic modulations of both spatial and recognition working memory (Shen et al. 1996; Everitt and Robbins 1997; Tang et al. 1997; Ragozzino and Kesner 1998; Warburton et al. 2003; Chudasama et al. 2004) and are potential sites of interactions between RXRγ and cholinergic system. Finally, a recent report that mice carrying null mutation of GluR1 subunit of AMPA receptor display similar to our mutants deficits of spatial working memory (Reisel et al. 2002) incites us to search for possible links between retinoids and glutamatergic signaling, one of the fundamental elements involved in the mechanisms of learning and memory.

The present implication of RXRγ in the control of working memory is the first evidence for a specific and nonredundant function of this receptor, which does not play any apparent role during mouse development (Krezel et al. 1996), and the role of which in the control of locomotor functions, has been shown to be redundant with RXRβ (Krezel et al. 1998). Thus, the brain-specific expression of RXRγ, which was acquired in the course of evolution relatively recently during the transition between birds and rodents (Rowe et al. 1991; Krezel et al. 1999), may suggest that this receptor has acquired new function(s) in higher vertebrates, possibly unique to more complex brain functions, such as specific types of learning and memory processes. Finally, the lack of synergy between mutations of RARβ and RXRγ in the generation of the present behavioral phenotype, which is surprising, as RARβ and RXRγ are often coexpressed in the mouse brain, may suggest that either RXRγ modulates mnemonic functions in form of heterodimers with: (1) another RAR(s), and thus, is implicated in RA signaling or, (2) other nuclear receptors that are known to heterodimerise with RXRs, e.g., thyroid receptors, peroxisomal proliferator-activated receptors, or orphan receptors such as Nurr1 and NGFI-B (Mangelsdorf and Evans 1995; Chambon 1996; Wallen-Mackenzie et al. 2003). Such interactions may directly implicate other signaling pathways in control of cognitive functions.

Thus, the present study provides a new genetic animal

model for studying the mechanisms of mnemonic functions, which may be of relevance in research on neurological diseases or psychiatric disorders characterized by deficits of working memory. In fact, such deficits are the fundamental feature in schizophrenia, a disorder that shows several additional links with retinoids. In particular, retinoids are important modulators of relational memory in rodents (Etchamendy et al. 2001, 2003), which is also affected in schizophrenia (Titone et al. 2004). Retinoid receptors also appear to be important modulators of dopaminergic neurotransmission (Samad et al. 1997; Krezel et al. 1998), one of the key neurotransmission systems implicated in the aetiology of the disease. Furthermore, alterations of retinoid signaling during embryonal development lead to a number of congenital malformations, some of which are also encountered in the schizophrenic syndrome (Goodman 1998). Finally, a number of genes coding for proteins involved in retinoid signaling colocalize also with loci mapped for schizophrenic syndromes (Goodman 1998), including locus containing the RXRγ gene (Lewis et al. 2003, and references therein).

Materials and Methods

Animals

Single RARβ$^{-/-}$ and RXRγ$^{-/-}$ mutants were generated as described (Krezel et al. 1996; Ghyselinck et al. 1997). Crosses of double heterozygotes were used to obtain RARβ$^{-/-}$/RXRγ$^{-/-}$ double mutants, called hereafter RARβ/RXRγ mutants, corresponding single mutants, and control wild-type groups. These experimental animals were also generated from crosses among null mutants or among wild-type mice of the same genetic background, and were used in tests as indicated below. However, since they were not different from mice originating from heterozygous crosses, they were not shown separately. Male mice aged between 4 and 5 mo were used in tests, and their genetic background was 60% C57/B6j and 40% 129/SVpas. All mice were housed in 7 am–7 pm light/dark cycle in individually ventilated cages, type "MICE" (Charles River). Food and water were freely available unless otherwise indicated, and all tests were performed between 8 a.m. and 4 p.m. All experiments were carried out in accordance with the European Community Council Directives of 24 November 1986 (86/609/EEC) and with the guidelines of CNRS and the French Agricultural and Forestry Ministry (decree 87848).

Cross-maze based tests

Apparatus

The apparatus used was a four-arm radial maze (cross-maze) made of PVC with a gray floor and translucent walls. It consisted of a central square platform (9 × 9 cm) and four arms (40-cm long, 9-cm wide, and 16-cm high). Each arm contained at its extremity a tray in which a drop of 25% sucrose was placed according to conditioning protocol. A sliding shutter made of gray Plexiglas was used to block one of the four arms, so that the maze was used as a T-maze in delayed nonmatch to place or right/left discrimination tests. Two other shutters were used to release an animal from the starting box and to block it after the arm entry. Urine and feces were cleaned between each animal with 10% alcohol solution.

Habituation

Forty eight hours before habituation, animals were water deprived during the night, and this deprivation was repeated throughout the entire training. Animals had access to water only 2 h per day after the habituation or test session. On the second day of water deprivation, animals were given access to 25% sucrose in their home cages in order to habituate to the reinforcement.

Habituation to the apparatus was carried out on two consecutive days. For each habituation session, mice were separated in single cages 30 min prior to the test, and then, each mouse was placed in the middle of the cross-maze and allowed to visit the maze freely for a minimum of 5 min, with a 10-min cut-off period. During this time, the animals had to visit all arms and drink drops of sucrose water (25%), which were dispensed in each of the four trays.

Delayed nonmatch to place

The test was used as previously described (Chapman et al. 1999). Behaviorally naive groups of 17 wild-type (seven derived from homozygous crosses) and 16 RARβ/RXRγ (six derived from homozygous crosses) mutant mice and seven RARβ, seven RXRγ mutant mice, and seven wild-type controls, respectively, were tested in the DNMTP in the T-maze over nine consecutive days. During this time, control mice attained the criterion performance, which was set at a minimum of 90% of correct choices over three consecutive days. Each daily training consisted of six trials, each trial being composed of two phases, acquisition and retention phase (Fig. 6). Thus, each day, each animal was exposed 12 times to the apparatus. At the beginning of each trial, one drop of 25% sucrose was deposited in the wells present in the two opposing arms. For the acquisition phase, one of these arms was blocked and the mouse was placed in the start box, which was always positioned at the base of the T-maze. After 15 sec, the mouse was released, and the time to enter the opened arm, "forced latency," was measured. The mouse was blocked in the arm on its entrance. After drinking the sucrose reward, it was transferred into the start box and both arms were opened. The time between the acquisition and retention phase during each trial was maintained at 10–15 sec; however, since during this time each mouse was kept in the start box without changing the context, we considered this delay as minimal "0 sec" delay. After removing the shutter of the start box, the animal was allowed to enter the arm of choice, where it was blocked on entrance. A choice was rewarded and considered as correct if the animal entered the arm not visited during the acquisition phase. After consuming the sucrose, or after 30 sec, if this arm was not baited, the animal was returned to its isolation cage. For each animal, the number of correct choices and the latency to enter the visited arm during each trial were recorded. Trials were separated by a 15–20-min interval.

Place reference test in a cross–maze

The place reference test and right/left discrimination were adapted from Thompson et al. (1980). Training was carried out for 9 d, and each day, animals received 12 trials semirandomly organized into four trials, starting from one of three starting arms (similar to design illustrated previously) (Ragozzino et al. 1999a). For each trial, the baited arm remained the same for an individual mouse throughout the entire experiment and was randomized for each group among south, west, north, or east. The mouse was placed in the start box of the designated arm for 15 sec, and then allowed to choose one of the two other arms; only one arm was reinforced with 25% sucrose. Once the animal chose one of the two arms, the arm was blocked. After consuming the sucrose, or after 30 sec, if this arm was not baited, the animal was returned to its isolation cage. Trials were separated by a 15–20-

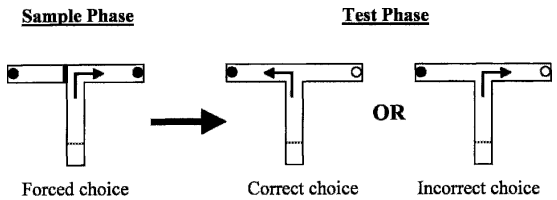

Figure 6. Delayed nonmatch to place paradigm. During the acquisition phase, the mouse is forced to turn to the right (as illustrated) or left arm, where it finds the sucrose reward. During the retention phase, the reward is present in the arm not visited during the acquisition phase, and its finding is considered a correct choice. (●) The well containing sucrose reward.

min interval. For each animal, the number of correct choices and the latency to enter the arms during each trial were recorded.

Right–left discrimination in a cross–maze

Training was carried out for 9 d, and each day, the animals received 12 trials semirandomly organized into three trials, starting from one of four starting arms (south, west, north, or east; similar to design illustrated previously) (Ragozzino et al. 1999a). For each trial, only three arms, in the form of a letter T, were used with the start box chosen at the base of the T-maze. The mouse was placed in the start box of the designated arm for 15 sec, and then allowed to explore freely the two other arms; only one arm was reinforced with 25% sucrose. Once the animal chose one of the two arms, the arm was blocked. After consuming the sucrose, or after 30 sec, if this arm was not baited, the animal was returned to its isolation cage. Each animal was reinforced consequently in the right or the left arm throughout the entire experiment, and each experimental group was randomized with respect to reinforcement in the right and left arm. The start-box arm was changed from trial to trial so that animals would not be able to use extra-maze cues to solve the task, and instead, had to use only egocentric cues to perform correctly in this test. Trials were separated by a 15–20-min interval. For each animal, the number of correct choices and the latency to enter the arms during each trial were recorded.

Data analysis

Since values corresponding to a percent of correct choices range from 0% to 100% and do not follow normal distribution, we have used angular transformation [2*asin(√value)] before any further analysis of these data. A global analysis of data was made using ANOVA on repeated measures with genotype as a between factor, and training as a within factor. In single-mutant studies, the post-hoc comparisons were done using the Dunnett test, whereas whenever only two groups were compared for individual time points, we used unpaired Student *t*-test.

Y-maze spontaneous alternation

The Y-maze spontaneous alternation paradigm is based on the natural tendency of rodents to explore a novel environment. When placed in the Y-maze, mice will explore the least recently visited arm, and thus tend to alternate visits between the three arms. For efficient alternation, mice need to use working memory, and thus, they should maintain an ongoing record of most recently visited arms, and continuously update such a record. A mouse with an impaired working memory cannot remember which arm it has just visited, and thus shows decreased spontaneous alternation (Holcomb et al. 1999; Wall and Messier 2002).

The Y-maze apparatus, made of Plexiglas had three identical arms (40 × 9 × 16 cm) placed at 120° with respect to each other. Specific motifs were placed on the walls of each arm, thus allowing visual discrimination, although extra-maze cues of the room were also visible from the maze. We used behavioraly naive RARβ/RXRγ (n = 10) double-mutant mice with wild-type controls (n = 12) and RARβ (n = 12) and RXRγ (n = 12) single-mutant mice, with corresponding wild-type control group (n = 14). Each mouse was placed at the end of one arm and allowed to explore freely the apparatus for 5 min, with the experimenter out of the animal's sight. Spontaneous alternation performance (SAP) was assessed visually by scoring the pattern of entries into each arm during the 5 min of the test. Alternations were defined as successive entries into each of the three arms as on overlapping triplet sets (i.e., ABC, BCA, . . .). Percent of spontaneous alternation was defined as the ratio of actual (= total alternations) to possible (=total arm entries −2) number of alternations × 100. The alternate arm returns (AARs) and same arm returns (SARs) were also scored for each animal in order to assess aspects of attention within spontaneous working memory (Wall and Messier 2002). Total entries were scored as an index of ambulatory activity in the Y-maze, and mice showing scores below six entries would be excluded. A global analysis of data was made using ANOVA. In single-mutant studies, the post-hoc comparisons were done using the Dunnett test, whereas comparisons between two groups for individual time points were done using the unpaired Student *t*-test.

Object-recognition task

The object-recognition task (Ennaceur and Delacour 1988) is based on the natural tendency of rodents to explore a novel object/environment and to compare it with a familiar one. The test was performed in an open field (44 × 44 × 18 cm) made of PVC with a black floor and translucent walls (Panlab). The open-field was placed in a homogeneously illuminated room (70 lux at the level of the open field) and the objects to be discriminated were a glass marble (2.5-cm diameter) and a plastic dice (2 cm). Animals were first habituated to the open-field for 30 min. The next day, they were submitted to a 10-min acquisition trial (first trial), during which they were individually placed in the open-field in the presence of an object A (marble or dice) placed semirandomly in one of the two presentation positions (in the corner, 10 cm from side walls). The time taken by the animal to explore the object A (when the animal's snout was directed toward the object at a distance ≤1 cm) was manually recorded. Mice that explored object A for <4 sec were excluded. Remaining animals were divided into the following groups to be tested only one time in the 10-min retention trail (second trial) that occurred 1, 3, or 24 h later. Thus, at the 1-h delay, we tested n_{WT} = 10 and $n_{RAR\beta/RXR\gamma}$ = 10; at a 3-h delay n_{WT} = 20 (nine derived from homozygous crosses) and $n_{RAR\beta/RXR\gamma}$ = 20 (10 derived from homozygous crosses) and at 24 h n_{WT} = 10 and $n_{RAR\beta/RXR\gamma}$ = 10 mice. In the study of single mutants for 3-h delays, we used $n_{RAR\beta}$ = 8, $n_{RXR\gamma}$ = 21 (nine derived from homozygous crosses) and n_{WT} = 10 mice. Due to low exploratory activity (<4 sec of exploration), we had to exclude from analysis, in total, five wild-type, two RARβ/RXRγ, one RARβ, and two RXRγ mice. These animals were not considered for the group sizes indicated above. During the retention test, the duplicate of object A and another object B (marble or dice, different from object A) were placed semirandomly in two presentation positions on the opposite sides of the open field (in the corners, 10-cm from side walls), different from the position used during the acquisition phase, and the times t_A, and t_B taken by the animal to explore the two objects were recorded. A recognition index (RI) was defined as $[t_B/(t_A + t_B)] \times 100$. The type of the objects and their positions of presentation during acquisition and retention phase were counterbalanced across animals. To prevent olfactory cues, objects were washed with ethanol after each test, and forceps were used to place the objects in the arena. A global analysis of data was made using ANOVA. In single-mutant studies, the post-hoc comparisons were done using the Dunnett test, whereas comparisons between two groups for individual time points were done using unpaired Student *t*-test.

Immunodetection of RXRγ protein

Coronal sections (14-μm thick) from unfixed frozen brains of three 3-mo old males (F_1 cross between C57/B6j × 129Sv/pas, Charles River) were collected on superfrost slides, and stored at −80°C until analysis. For analysis of RXRγ expression, sections were post-fixed in 4% paraformaldehyde and treated with 1% H_2O_2 to block endogenous peroxidase. Rabbit anti RXRγ polyclonal antibody (1:500, Santa Cruz Biotech) was used for immunodetection of the protein and was revealed using the ABC system (Vector) according to the manufacturer's manual. Brain regions expressing RXRγ were identified according to the mouse-brain atlas (Paxinos and Franklin 2001). Brains from RXRγ mutant mice were used for negative control.

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